

## Heterotypic Humoral and Cellular Immune Responses following Norwalk Virus Infection<sup>▽†</sup>

Lisa C. Lindesmith,<sup>1</sup> Eric Donaldson,<sup>2</sup> Juan Leon,<sup>3</sup> Christine L. Moe,<sup>3</sup> Jeffrey A. Frelinger,<sup>2</sup> Robert E. Johnston,<sup>4</sup> David J. Weber,<sup>5</sup> and Ralph S. Baric<sup>1,2,4\*</sup>

*Department of Epidemiology, University of North Carolina, Chapel Hill, North Carolina<sup>1</sup>; Department of Microbiology and Immunology, University of North Carolina, Chapel Hill, North Carolina<sup>2</sup>; Hubert Department of Global Health, Emory University, Atlanta, Georgia<sup>3</sup>; Carolina Vaccine Institute, Chapel Hill, North Carolina<sup>4</sup>; and Department of Medicine, University of North Carolina, Chapel Hill, North Carolina<sup>5</sup>*

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Norovirus immunity is poorly understood as the limited data available on protection after infection are often contradictory. In contrast to the more prominent GII noroviruses, GI norovirus infections are less frequent in outbreaks. The GI noroviruses display very complex patterns of heterotypic immune responses following infection, and many individuals are highly susceptible to reinfection. To study the immune responses and mechanisms of GI.1 persistence, we built structural models and recombinant virus-like particles (VLPs) of five GI strains: GI.1-1968, GI.1-2001, GI.2-1999, GI.3-1999, and GI.4-2000. Structural models of four GI genotype capsid P domain dimers suggested that intragenotype structural variation is limited, that the GI binding pocket is mostly preserved between genotypes, and that a conserved, surface-exposed epitope may allow for highly cross-reactive immune responses. GI VLPs bound to histo-blood group antigens (HBGAs) including fucose, Lewis, and A antigens. Volunteers infected with GI.1-1968 ( $n = 10$ ) had significant increases between prechallenge and convalescent reactive IgG for all five GI VLPs measured by enzyme immunoassay. Potential cross-neutralization of GI VLPs was demonstrated by convalescent-phase serum cross-blockade of GI VLP-HBGA interaction. Although group responses were significant for all GI VLPs, each individual volunteer demonstrated a unique VLP blockade pattern. Further, peripheral blood mononuclear cells (PBMCs) were stimulated with each of the VLPs, and secretion of gamma interferon (IFN- $\gamma$ ) was measured. As seen with blockade responses, IFN- $\gamma$  secretion responses differed by individual. Sixty percent responded to at least one GI VLP, with only two volunteers responding to GI.1 VLP. Importantly, four of five individuals with sufficient PBMCs for cross-reactivity studies responded more robustly to other GI VLPs. These data suggest that preexposure history and deceptive imprinting may complicate PBMC and B-cell immune responses in some GI.1-1968-challenged individuals and highlight a potential complication in the design of efficacious norovirus vaccines.

Noroviruses are the second-most important cause of severe viral gastroenteritis in young children and cause approximately 20% of endemic familial diarrheal disease and traveler's diarrhea in all ages (reviewed in references 45 and 70). Noroviruses are genetically grouped into five different genogroups (GI to GV). GI and GII genogroups are responsible for the majority of human infections and are subdivided into more than 25 different genotypes (for example, GI.1 is genogroup I genotype 1). Most norovirus outbreaks are caused by the GII.4 genotype (65). Although genogroup I strains are associated with fewer reported outbreaks, they are frequently identified in environmental samples and in children (7, 21, 33, 58, 74, 82). The severity of norovirus disease is usually moderate although infection can be especially virulent, even fatal, in the elderly (14, 24, 31, 38, 46, 67). An effective vaccine would be particularly advantageous to vulnerable older populations, food handlers,

child and health care providers, and military personnel. One major obstacle to norovirus vaccine development is the lack of understanding of the extensive antigenic relationships between heterogenic norovirus family members and of how this antigenic heterogeneity affects host protective immunity. Norovirus heterogeneity can be examined through sequence, structural, ligand binding, and host immune studies.

Structurally, noroviruses are ~38-nm icosahedral viruses with an ~7.5 kb single-stranded, positive-sense RNA genome that encodes three large open reading frames (ORFs). ORF1 encodes the replicase polyprotein, while ORFs 2 and 3 encode the major and minor capsid proteins, respectively. The ORF2 major capsid protein sequence can vary by up to 60% between genogroups and by ~20 to 30% between the genotypes (91). Expression of the major capsid protein (ORF2) in baculovirus and Venezuelan equine encephalitis (VEE) results in formation of virus-like particles (VLPs) composed of 180 copies of the monomeric protein (72). The monomer is structurally divided into the shell domain (S) that forms the structural core of the particle and the protruding domain (P) that protrudes away from the core. The P domain is further subdivided into the P1 subdomain (residues 226 to 278 and 406 to 520) and the P2 subdomain (residues 279 to 405) (72). P2 represents the most exposed surface of the viral particle and determines in-

\* Corresponding author. Mailing address: 2103 McGavran-Greenberg HA, CB7435, School of Public Health, University of North Carolina—Chapel Hill, Chapel Hill, NC 27599. Phone (919) 966-3895. Fax: (919) 966-2089. E-mail: rbaric@email.unc.edu.

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teraction with both potential neutralizing antibody recognition sites and putative cellular receptors, the histo-blood group antigens (HBGAs) (13, 16, 54, 57).

The P domain has been shown to independently form dimers and P particles comprised of 12 monomers (85). Dimers and P particles share structural and HBGA binding similarities with the VLP generated with the same monomers (9, 85, 87). Three norovirus-HBGA binding profiles have been identified: (i) those that bind A/B and/or H epitopes, (ii) those that bind Lewis and/or H epitopes, and (iii) those that do not bind any available HBGA (86). Elegant structural analyses of Norwalk virus VLPs in complex with synthetic HBGAs identified a highly conserved binding site within the G1 noroviruses and predicted that structural constraints within the GI strains would restrict HBGA binding patterns to either a terminal Gal-Fuc or GalNAc (18, 88).

Norwalk virus (NV; GI.1-1968) is the prototypic GI strain and typically infects individuals who encode a functional *FUT2*  $\alpha$ -1,2-fucosyltransferase enzyme resulting in expression of HBGAs on mucosal surfaces (secretor-positive phenotype) (53). Individuals who do not encode a functional *FUT2* enzyme have a secretor-negative phenotype, do not express ABH HBGAs on mucosal surfaces, and are resistant to NV infection. Outbreak investigations have confirmed the association between HBGA expression and norovirus infection for some GI and GII strains (37, 39, 43, 49, 89). It remains likely that enzymes other than *FUT2* may function as norovirus susceptibility factors because secretor-negative individuals have low-level norovirus-reactive antibodies (49, 52, 53) and can become infected after challenge with a GII.2 strain (52); in addition, some norovirus strains bind to *FUT2*-independent HBGAs *in vitro* (35, 54, 79).

Early challenge studies (reviewed in reference 50) suggested that short-term protective immunity may occur following NV challenge (96). Demonstration of long-term protective immunity has been more complex. One early rechallenge study found that 50% of NV-challenged volunteers experienced repeat infections after ~3 years while the other 50% remained well initially and after repeated challenge (69). Whether these volunteers remained disease free because of acquired immunity or genetic resistance could not be ascertained (69). However, contemporary norovirus challenge studies suggest that an early mucosal IgA response is associated with protection from NV infection (53). Further, strong gamma interferon (IFN- $\gamma$ ) secretion from CD4<sup>+</sup> T cells (52) was identified in some uninfected GII.2-1976-challenged volunteers.

In the absence of additional rechallenge studies, the most compelling evidence for a long-term protective immune response comes from the growing number of reports from around the world indicating that periods of "high norovirus activity" correlated with the emergence of new GII.4 strains (1, 10, 42, 66, 75, 90). Subsequently, the years following the high activity were characterized by decreased numbers of outbreaks, indicating that herd immunity may be an important regulator of GII.4 noroviruses (54, 80, 81). Clearly, the molecular basis for differential protective immunity/susceptibility following repeat norovirus infection is complex and a major challenge for the field.

In this report, we compare the VLP phenotypes of the prototypical norovirus strain NV to an extant GI.1 strain isolated

33 years after NV and to a panel of VLPs representing strains GI.2, GI.3, and GI.4. In the results, we evaluate sequence conservation, carbohydrate (CHO) binding patterns, and antigenic relatedness at the antibody and T-cell levels. In contrast to earlier predictions (19), these data suggest that the GI noroviruses can bind many different HBGAs and that individuals infected with norovirus usually mount robust B- and T-cell responses against homologous strains. Surprisingly, some individuals appear to preferentially mount immune responses against heterologous GI strains.

## MATERIALS AND METHODS

**Ethics statement.** Archived serum samples were collected from individuals infected with GI.1-1968 for an unpublished pilot study (C. L. Moe, unpublished data) conducted with the volunteers' written informed consent and with approval from the IRBs at their respective institutions.

**Phylogenetic analysis of the norovirus GI ORF2 sequences.** At the time of this study, 44 full-length and unique GI genotype capsid sequences were available at NCBI, and these amino acid sequences were downloaded and included in a collection with four of our own in-house sequences, for a total of 48 full-length sequences (see Table S1 in the supplemental material). Additional sequences were present but were identical to sequences selected in the final unique set. The 48 unique amino acid sequences were aligned by ClustalX, version 1.83 (17), using the percent accepted mutation (PAM) distance matrix and default parameters (see Fig. S1 in the supplemental material). The alignment was refined manually, and sites of variation, defined as any site with a quality score of less than 100, were exported in table format and ordered by genotype. To eliminate potential sequencing errors, positions that were different in only one representative sequence were removed. Variable sites that occurred in the P2 subdomain were exported using Microsoft Excel.

Phylogenetic analysis was conducted using amino acid alignment to generate a phylogenetic tree via Bayesian inference (BI) using Mr. Bayes, version 3.12 (76). Briefly, the alignment was exported in the nexus format, the amino acid substitution model was set to Dayhoff (22) using the *lset* command, and Markov chain Monte Carlo simulation (27, 32, 36) was used to approximate the posterior probabilities of trees, with sampling conducted on four chains over 500,000 generations (77). Trees were sampled every 100 generations, and the 5,001 trees collected were summarized with the *sumt* command set to a burn-in of 1,000, which generated a consensus tree using the 50% majority rule (77). The burn-in value was determined using the *sump* command with an arbitrary burn-in of 250, which demonstrated that stationarity occurred prior to the 100,000th generation, indicating that a burn-in of 1,000 was appropriate for the *sumt* command (77).

**Mapping informative sites onto the predicted structure.** The X-ray crystal structure of the P domain of GI.1 Norwalk virus in complex with H type 1-pentasaccharide and A-trisaccharide (19) (Protein Data Bank [PDB] accession numbers 2ZL5, 2ZL6, and 2ZL7) was used as a template to generate comparative homology models of each GI genotype sequence using the program 3D-Jigsaw (3, 4, 20), with default parameters, and Modeler, version 8.2, using the auto-model class (26, 60). Five models of each were generated, and the model with the lowest objective function score was selected for analyses. The PDB files generated by these programs were visualized using the molecular modeling tools MacPyMol (DeLano Scientific) and Chimera (71). In addition, the variation measured in the multiple sequence alignment was mapped onto the Norwalk VLP structure (PDB code 1IHM) using the program ConSurf, version 3.0, to generate a structure highlighting the variable sites (47).

**GI VLPs.** Capsid gene constructs for each strain were designed and synthesized as reported previously (2, 54). Briefly, the ORF2 genes of GI.1-1968 (2), GI.2-1999, GI.3-2000, and GI.4-2000 were derived from reverse transcription-PCR (RT-PCR) products from outbreak stool samples (54, 92) while the ORF2 gene of GI.1-2001 (accession number AY502016) was synthesized commercially by BioBasic (<https://www.biobasic.com/index.php>) (54). All ORF2 constructs were then inserted directly into the VEE replicon vector for the production of virus replicon particles (VRPs). VLPs were expressed in VRP-infected BHK cells and purified by velocity sedimentation in sucrose, and approximately 35-nm particles were visualized by negative-staining electron microscopy (EM) (2, 56). VLP concentrations were determined by a Bio-Rad protein assay (Bio-Rad, Hercules, CA) and 1  $\mu$ g/ml was used to coat enzyme immunoassay (EIA) plates, bind HBGAs, and stimulate peripheral blood mononuclear cells (PBMCs).

**Carbohydrate binding assays.** VLP binding to HBGA-phenotyped boiled salivary samples and synthetic HBGAs was determined as reported previously by

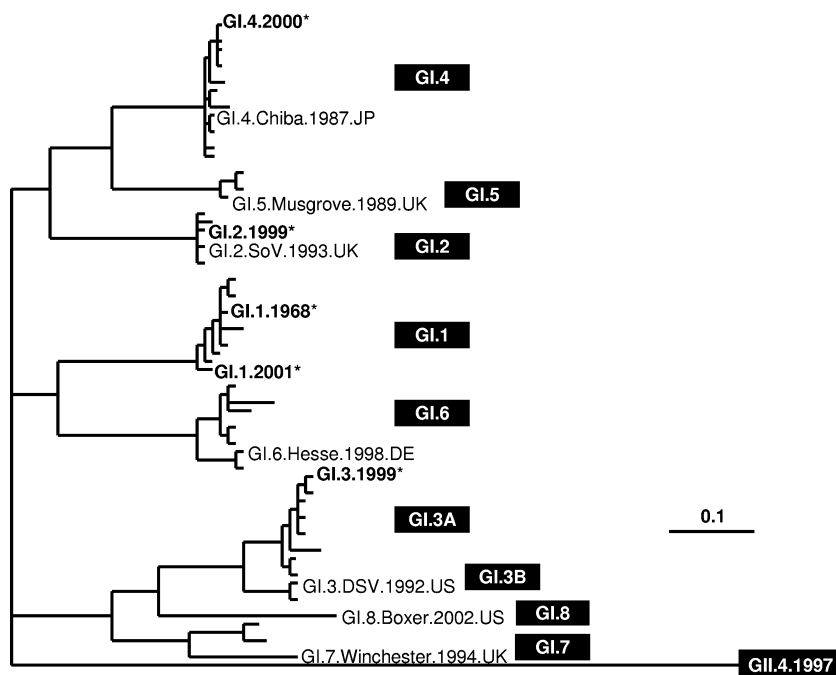


FIG. 1. Bayesian analysis of the GI genogroup. The 48 sequences of the GI genogroup were aligned and analyzed by Bayesian inference, which demonstrated the evolutionary relationship between the eight genotypes. Only the reference sequences are shown, along with those sequences used to generate VLPs, indicated in bold with an asterisk. Black boxes indicate genotypes. Scale bar, 0.1 substitutions per site.

our group (52, 53) with the exception of the use of either mouse anti-VLP antiserum (either strain-specific or cocktails) or rabbit anti-GI VLP polyclonal serum, followed by anti-mouse/rabbit alkaline phosphatase (Sigma Aldrich, St. Louis, MO) and *p*-nitrophenyl phosphate (pNPP; Sigma Aldrich) for VLP binding detection. Blockade experiments used H type 3 for GI.1-1968, GI.1-2001, and GI.2-1999 and Le<sup>a</sup> for GI.3-1999 and GI.4-2000 HBGA ligands. VLP binding to synthetic HBGA was determined using NeutriAvidin-coated plates (Pierce, Rockford, IL) treated with 10  $\mu$ g/ml biotinylated carbohydrate (Glycotect, Gaithersburg, MD, and the Consortium for Functional Glycomics, grant number GM62116) for 1 h and washed with phosphate-buffered saline (PBS)-0.05% Tween 20 before the addition of 1  $\mu$ g/ml VLP for 1.5 h. VLP binding was detected with rabbit anti-GI VLP polyclonal serum followed by pNPP. All incubations were done at room temperature. The 50% blockade titer (BT50) was defined as the reciprocal of lowest serum dilution tested that blocked at least 50% of binding compared to levels determined in the absence of antibody pretreatment. Serum samples that did not reach a BT50 by the maximum percent serum tested were assigned a BT50 value equal to the twice the maximum percent serum tested for statistical analysis. Serum samples that blocked >50% binding at the lowest percent serum tested were assigned a BT50 value equal to one-half the minimum percent serum tested for statistical analysis (54).

**Serum samples.** Volunteers were determined to be infected with GI.1-1968 by the sequence of the challenge inoculum and the presence of GI.1-1968 RNA in any postchallenge stool sample or by seroconversion to GI.1-1968. Seroconversion was defined as a  $\geq 4$ -fold increase above prechallenge (day 0) titer in postchallenge (day 14) samples (53). Geometric mean titers of VLP-reactive serum IgG were measured by EIA (52-54). Briefly, plates were coated at 1  $\mu$ g/ml VLP in PBS for 4 h at room temperature and blocked overnight at 4°C in 5% Carnation dry milk in PBS-0.05% Tween 20 before the addition of serially diluted serum. The serum was incubated for 1 h at 37°C before addition of mouse anti-human IgG-alkaline phosphatase (Sigma Chemicals) for 30 min at 37°C and color development with pNPP (Sigma Chemicals). Each step was followed by washing with PBS-0.05% Tween 20, and all antibodies were diluted in 5% Carnation dry milk in PBS-0.05% Tween 20. Anti-VLP serum IgG was compared to a purified IgG (Sigma Chemicals) of known concentration for quantification. In this report archived plasma pairs obtained from 10 individuals infected with GI.1-1968 in an unpublished pilot study (Moe, unpublished) are characterized. Additional serum samples collected during a reported GII.2-1976 human challenge study (52) and a GII.4 outbreak (54) were used for comparisons.

**VLP stimulation of PBMCs.** The 10 infected volunteers who provided serum samples also provided PBMCs collected prechallenge (day 0) and postchallenge (days 4, 14, and 35). Cryo-preserved PBMCs were processed, aliquoted at  $5 \times 10^5$  cells/well, and stimulated with either 10 ng/ml phorbol myristate acetate (PMA)-0.5  $\mu$ g/ml ionomycin, medium only, or 1  $\mu$ g/ml GI VLP for 48 h, as described previously (52). Cell-free culture supernatants were collected and stored at -20°C until evaluation of secreted IFN- $\gamma$ . A significant percentage of the volunteers (30%) did not have prechallenge samples for comparison. To mitigate the impact of missing samples and of hyperactivation in a few select archived samples, a more conservative definition was used for determining a PBMC response. PBMCs were considered responsive to a VLP if two consecutive postchallenge samples had a  $\geq 4$ -fold increase above the earliest collected sample (day 0 or day 4) (94).

**Measurement of IFN- $\gamma$  secretion.** IFN- $\gamma$  secretion was measured in duplicate by EIAs (BD Biosciences), and the lower limit of detection was 4 pg/ml. Samples below this limit were assigned a value equal to half the lower limit of detection (2 pg/ml) for analysis purposes (52). For all samples, the medium-stimulated background was subtracted.

**Statistical analysis.** IgG data were not normally distributed even after transformations; thus, the Kruskal-Wallis test, followed by a posthoc Dunn's multiple comparison test (KW test), was used for comparisons of IgG and blockade response between multiple groups. A Mann-Whitney two-tailed test (M-W test) was used to compare IgG responses between two groups (52). *P* values of less than 0.05 were considered significant.

## RESULTS

**GI sequence variation and phylogeny.** Based on the nucleotide sequence of ORF2, the major capsid protein gene, genogroup I noroviruses are currently subdivided into eight genotypes (97). All eight genotypes were represented in the 48 full-length capsid amino acid sequences analyzed in this study. Bayesian analysis, using a GII.4 sequence as an outgroup, was used to generate a phylogenetic tree showing the evolutionary relationships of these viruses (Fig. 1). In contrast to findings reported among GII.4 noroviruses (54), sequence variation in

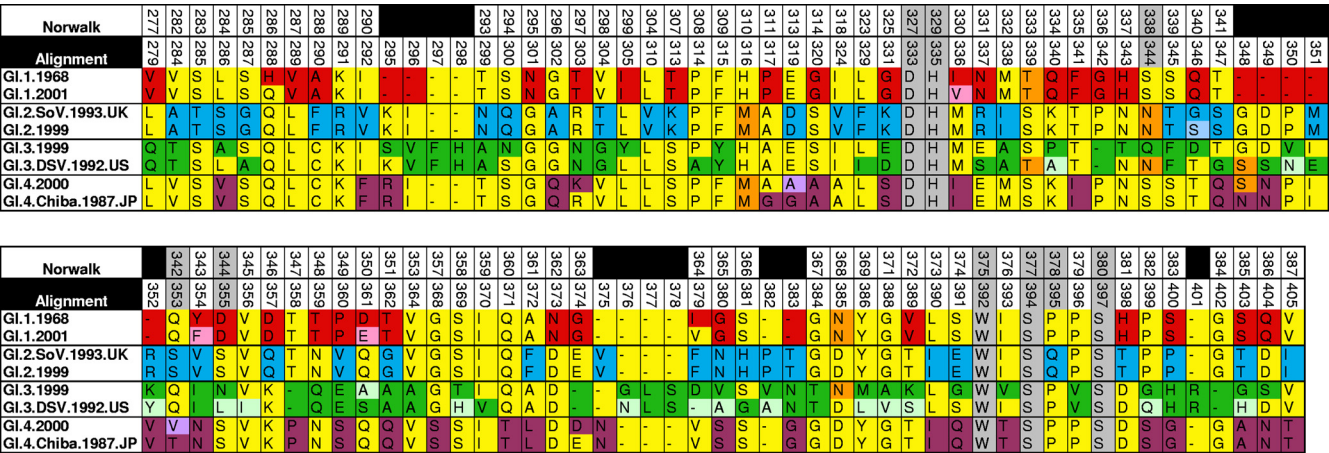


FIG. 2. Variation in the P2 subdomain of the first four GI genotypes. Variation within the P2 subdomain of eight GI.1 to GI.4 representative sequences is shown with the genotype-specific changes colored as follows: GI.1 Norwalk, red; GI.2 Southampton, blue; GI.3 Desert Shield, green; and the GI.4 Chiba, purple. Amino acids that occur within multiple genotypes on one occurrence (yellow) and multiple occurrences (orange) are shown. Unique differences that occur within a genotype are indicated by a lighter shade of the primary genotype color. Position numbers filled in gray indicate residues that interact with HBGAs in the GI.1 Norwalk capsid, and columns that are shown in gray indicate residues that are strictly conserved within these four genotypes. Alignment indicates numbering based upon the multiple alignment, and Norwalk indicates numbering based upon the GI.1 Norwalk sequence. Representative sequences were selected to demonstrate the sequence heterogeneity within the genotype.

the tree occurred primarily between genotypes, with very little variation identified within any specific genotype (Fig. 1). Among these, the GI.3 genotype contained the most intragenotype variation showing two distinct clades, labeled A and B in Fig. 1. While variation occurred in the S domain and the P1 subdomain of the capsid, the most significant changes occurred in the P2 subdomain, consistent with results observed in the GII.4 genotype (54). Based on available VLPs, our study focused on the GI.1, GI.2, GI.3, and GI.4 genotypes. Analysis of these GI sequences in the P2 subdomain region demonstrated that significant variation occurred between genotypes in this subdomain although several of the residues that interact with the HBGAs (19) were strictly conserved within the GI genogroup (Fig. 2 and 3), as previously reported (19, 83). Of note, amino acids Gln353 and Asp355 (alignment numbering), which are present in the GI.1 genotype, have been shown to interact with the H type 1 pentasaccharide but not A trisaccharide in a crystallography study of GI.1 Norwalk VLP (9, 19). However, this motif was not conserved in the other GI noroviruses (9, 19), as Asp355 was present only in the GI.1 genotype sequences, which suggests that this negative charge may regulate GI.1 HBGA binding. Additionally, residues at alignment positions 344 and 353 may also play a role in regulation of carbohydrate binding (Fig. 2) as these sites are proximal to the binding site and are variable in some genotypes. In fact, S338 in the GI.1 genogroup (position 344 in the alignment) was shown to interact with the A trimer via a water bridge hydrogen bond (9, 88), and altering the amino acid at this position, as occurs in different genotypes, may result in differential HBGA binding. Interestingly, nine sites in the P2 subdomain were consistently different for each genotype, and these sites included 331, 352, 354, 360, 362, 380, 383, 400, and 403 (Fig. 2), using the alignment numbering as a reference.

**Structural variation within the GI genotype.** The Norwalk virus protruding domain structure (PDB 2ZL5) was used to generate homology models for genotypes: GI.2-1999 Southamp-

ton, GI.3-1999 Desert Shield, and GI.4-2000 Chiba sequences (Fig. 3). Comparison of these structural models with the Norwalk structure indicated that, while the variation in the P2 subdomain region altered the overall structure, many of these changes occurred away from the reported HBGA binding site (19) (Fig. 4). Several residues that were reported to interact with the HBGAs were strictly conserved (Fig. 4), and distal variation was predicted to have a less direct, perhaps regulatory, effect on binding. Within the GI.1 Norwalk genotype, variation between two isolates that were over 30 years apart resulted in only minor structural changes in the P2 subdomain as only five changes occurred in the P2 subdomain during this period. Of these five changes, only the change of Asp to Glu at position 361 of the multiple alignment (position 350 of Norwalk capsid) is predicted to result in minor structural differences on the surface of the capsid (Fig. 4A). Other changes within the genotype resulted in insertions of loops that extended to or beyond the surface (Fig. 4). In fact, GI.2, GI.3, and GI.4 models all had inserted loops that resulted in structural differences in the P2 subdomain while generally maintaining the topology of the HBGA binding pocket and introducing variation in the ridges surrounding the binding site (Fig. 4).

Analysis of the structure and mapping of the variation and insertions/deletions on the dimer models revealed that the variation that has occurred in the P subdomain between the different genotypes occurs primarily away from the binding site. Interestingly, a distinct conserved region that might include important epitopes for antibody recognition was identified in all four of the GI structures that we analyzed (Fig. 4). This region occurs distal to the binding site, and most of the conserved residues are surface exposed (Fig. 4). These conserved, surface-exposed residues may represent a common antibody epitope, conserved among the GI genogroup and perhaps facilitating the generation of cross-reactive antibodies

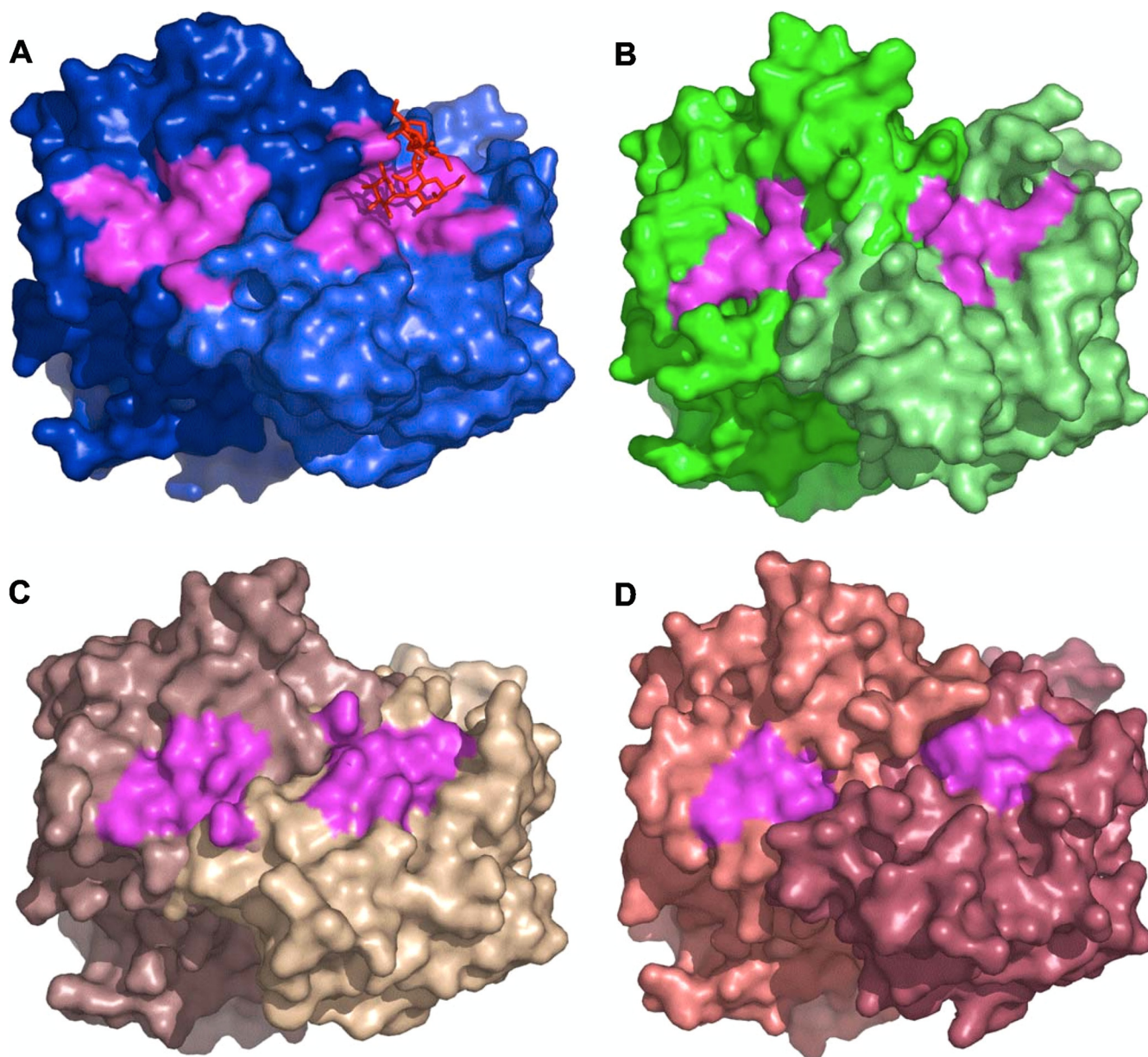


FIG. 3. Structural comparison between the Norwalk P domain structure and GI genogroup models. Homology models were generated for GI.1-2001, GI.2-1999, GI.3-1999, and GI.4-2000 P domain sequences using chain A and chain B of the Norwalk P domain dimer structure, and these were compared. In all cases, the residues reported to interact with the HBGAs were conserved (magenta). Chain A of each model is shown in the brighter shade on the left, and chain B is shown as the lighter shade on the right. Magenta indicates the binding pocket. (A) Norwalk crystal structure with the H type 1 pentasaccharide shown engaging with the receptor binding pocket. (B) The GI.2-1999 Southampton model shows that inserts have added loops to the top of the structure, which extends from the surface adjacent to the conserved binding pocket. (C) The GI.3-1999 Desert Shield model is structurally similar to that of the Norwalk structure, with a conserved receptor-binding pocket. (D) The GI.4-2000 Chiba model is also structurally similar to the Norwalk structure, with a conserved receptor-binding region.

against multiple GI noroviruses, as has been described in the literature (6, 44, 51).

**VLP production and characterization.** Genotypes GI.1, GI.2, GI.3, and GI.4 were available for VLP production to compare the antigenic relationship between GI genotypes. Across the genotypes, the capsid sequences share similarities ranging from 65 to 75%. To compare the antigenic relationship within a genotype, an additional GI.1-2001 VLP was prepared to compare it to GI.1-1968. Even with 30 years between collection

dates of these isolates, the capsid sequences of GI.1-1968 and GI.1-2001 strains are ~98% similar. All clones were confirmed to produce appropriately sized VLPs by electron microscopy before being characterized for carbohydrate binding ability and antigenicity.

Carbohydrate ligands were determined by binding to phenotyped saliva and to biotinylated synthetic HBGAs (54). All of the GI VLPs had preferential binding to secretor-positive, O and A blood type saliva, with more modest binding to B blood type saliva

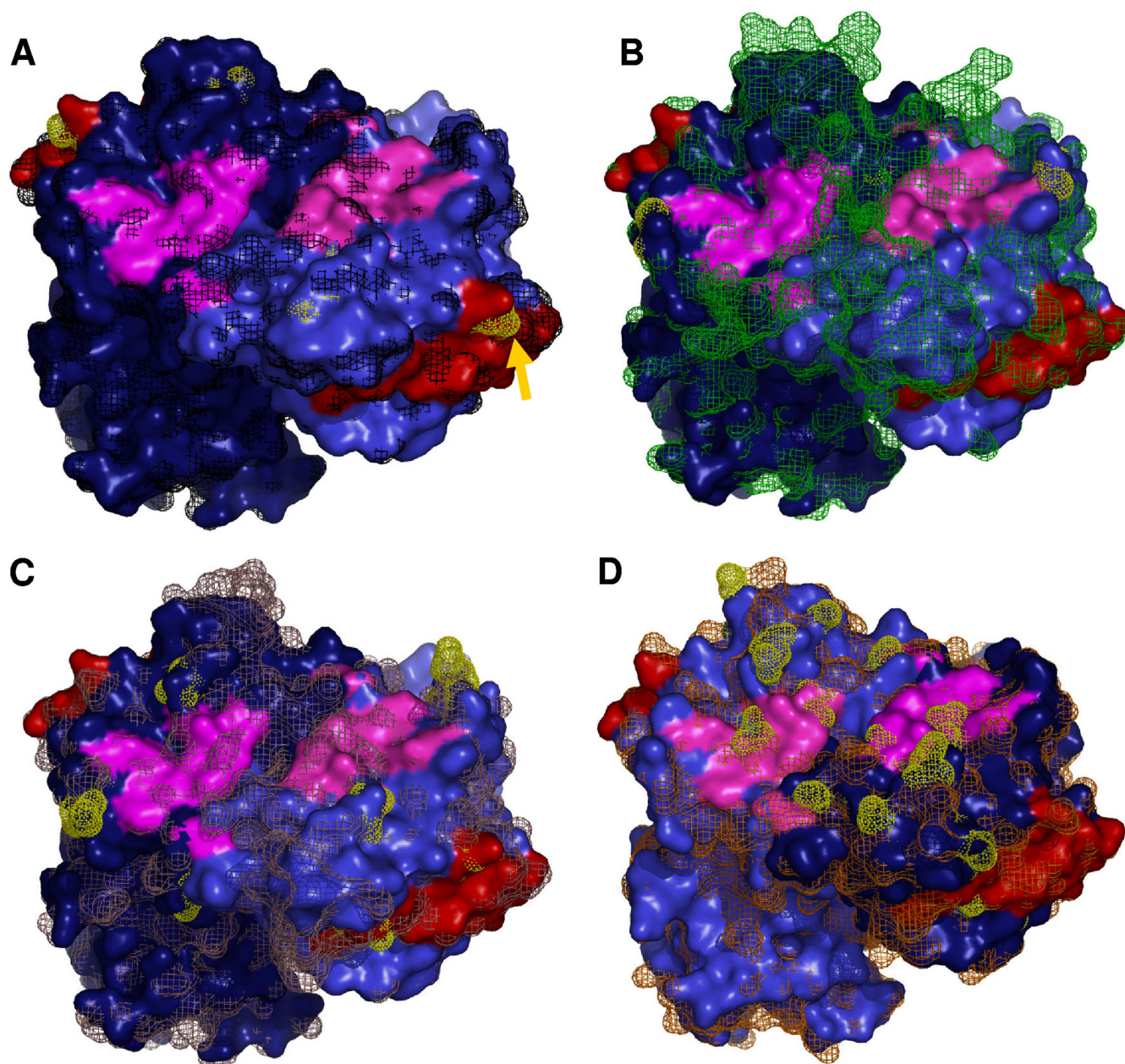


FIG. 4. Structural differences between the Norwalk P domain structure and the GI genogroup models. Analysis of the homology models in comparison to the Norwalk P domain dimer structure demonstrated that much of the variation in the genogroup occurs in loops that extend to the surface. Red indicates a conserved structural domain, purple indicates the binding pocket, and yellow indicates variation within the genotype. (A) The variation over 30 years within the GI.1 genotype resulted in only minor structural differences. The only observed change was a change of Asp to Glu at Norwalk capsid position 350 (arrow). (B) The GI.2-1999 Southampton genotype mostly differs from GI.1 by the addition of several residues that occur in loops shown in green. (C) The GI.3-1999 Desert Shield genotype mostly differs from GI.1 by the addition of residues in loops shown in brown. (D) The GI.4-2000 Chiba genotype mostly differs from GI.1 by the addition of only a few residues that occur in loops shown in orange.

at room temperature (Fig. 5). Low levels of binding to B blood type saliva are detected at room temperature but not 37° (data not shown), supporting previous reports of temperature-dependent VLP-ligand interaction (54, 78). GI.2-1999, GI.3-1999, and GI.4-2000 also bound to secretor-negative, Lewis-positive saliva. Neither of the GI.1 VLPs bound to secretor-negative saliva. Binding to synthetic biotinylated HBGAs confirmed these binding patterns (Fig. 6). Carbohydrate binding profiles between the two GI.1 VLPs was very similar. The GI.1 VLPs

bound to FUT2-dependent molecules H type 1, H type 3, and A trimer, as previously reported (34, 53, 59). The other GI VLPs all bound to FUT2 and/or Lewis-dependent carbohydrates. GI.2-1999 bound to H type 3 and Le<sup>a</sup>, GI.3-1999 bound only to Le<sup>a</sup>, and GI.4-2000 bound to Le<sup>a</sup>, Le<sup>x</sup>, and A trimer, supporting the novel observation of these GI VLPs binding to both secretor-negative and secretor-positive salivary samples.

**NV infection induces a GI broadly reactive antibody response.** Previous studies have indicated that the GI strains

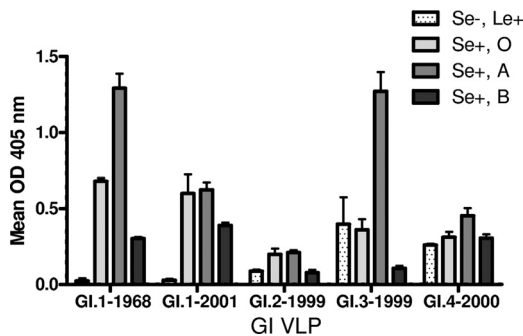


FIG. 5. GI VLP-salivary binding patterns at room temperature. GI VLPs were assayed for ability to bind to saliva samples phenotyped for secretor status (Se) and ABO blood type by EIA. OD, optical density.

share common antibody epitopes (25, 29, 68). The P2 domain is the most surface exposed part of the capsid, and antibody neutralization epitopes in noroviruses have been mapped to this region (57). An EIA was used to compare the antigenic relationship between NV GI.1-1968 and our panel of GI VLPs from 10 NV GI.1-1968-infected volunteers by using archived prechallenge (day 0) and convalescent-phase (day 14) sera. The percentage of subjects who had a  $\geq 4$ -fold increase between prechallenge and convalescent-phase serum samples (seroconverted) to each VLP and the median titer and range of prechallenge and convalescent-phase serum samples to each VLP are shown in Fig. 7. Prechallenge IgG titers were similar across the panel of VLPs (median, 10.5, 16.6, 30.6, 43.2, and 21.8  $\mu\text{g/ml}$  for NV GI.1-1968, GI.1-2001, GI.2-1999, GI.3-1999, and GI.4-2000, respectively). NV infection resulted in a significant increase in GI VLP-reactive IgG between prechallenge and convalescent-phase sera for all VLPs tested ( $P < 0.01$ , M-W test). Convalescent IgG titers were also similar between each VLP (median, 460.6, 452.3, 216.3, 614.0, and 389.5  $\mu\text{g/ml}$  for NV GI.1-1968, GI.1-2001, GI.3-1999, and GI.4-2000, respectively). Nine out of the 10 infected volunteers seroconverted to all five GI VLPs. The one exception was positive for GI.1-1968 RNA in multiple stool samples but did not seroconvert to any GI VLP except GI.2-1999. Prechallenge anti-GI VLP titers in this individual ranged from 47 times the group median for GI.1-1968 to 2.5 times for GI.2-1999. The elevated prechallenge titers were 496.2, 277.5, 75.3, 500.4, and 169.9  $\mu\text{g/ml}$  for NV GI.1-1968, GI.1-2001, GI.3-1999, and GI.4-2000, respectively.

**NV-induced IgG blocks ligand binding of multiple GI VLPs.** The high degree of serum cross-reactivity among the GI VLPs indicates that these strains share either many common or one/few immunodominant antibody epitopes. In the absence of a cell culture system for human norovirus cultivation, a surrogate assay for antibody-mediated neutralization was developed to evaluate this cross-reactive IgG in a functional assay. The blockade assay measures the ability of antisera to block the interaction of a VLP with a carbohydrate ligand (30, 54, 56). Although prechallenge serum samples reacted with each GI VLP, as a group they did not efficiently block the binding of any of the GI VLPs to HBGA. The dilution of serum needed to block 50% of binding (BT50 value) for each VLP was at or below the minimum dilution tested (BT50 of  $\leq 400$ ) (Fig. 8A). Binding of GI.4-2000 to HBGA was moderately inhibited in a nonspecific way, as evidenced by a decreased signal at all con-

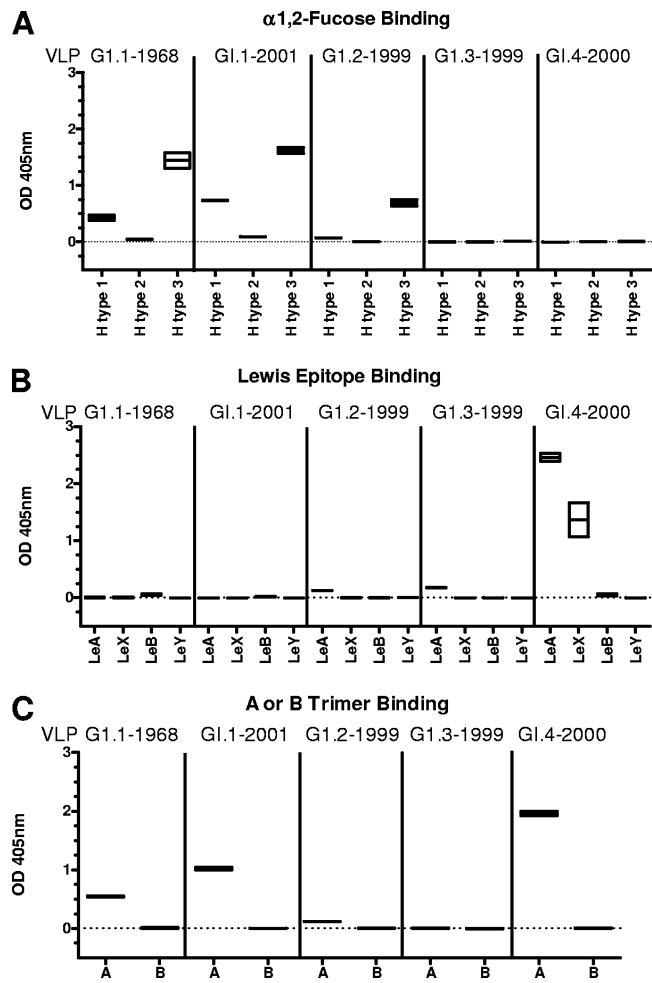


FIG. 6. GI VLP-carbohydrate binding patterns at room temperature. VLPs were assayed for the ability to bind to synthetic biotinylated HBGA bound to avidin-coated plates. The mean optical density (OD) is indicated by the line in the box. The upper and lower boundaries of the box represent the maximum and minimum values. (A) VLP binding to core chains including an  $\alpha 1,2$ -fucose. (B) VLP binding to either core chains or H antigens modified with the Lewis antigen. (C) VLP binding to A or B antigen trimer.

centrations studied without a dose dependence (Fig. 8A). Each volunteer had a unique prechallenge GI VLP blockade profile. Only two volunteers had detectable BT50s for GI.1-1968 in prechallenge serum (volunteers 09 and 10) (Fig. 9). These volunteers also had a prechallenge blockade antibody to the other GI VLPs. Volunteer 08 had prechallenge titers to GI.1-2000 and GI.3-1999. Regardless of prechallenge anti-NV GI.1-1968 IgG or blockade titer, GI.1-1968 infection resulted in an increased blockade ability for all five GI VLPs. As a group, convalescent-phase sera ( $n = 10$ ) effectively blocked GI.1-1968 interaction with H type 3, as well as GI.1-2001 and GI.2-1999 interaction with H type 3 and GI.3-1999 and GI.4-2000 interaction with Le<sup>a</sup> (Fig. 8B). The median BT50 serum dilution needed to block GI VLP-HBGA binding was 400 for GI.1-1968, which is similar to the BT50 for blockade of GI.1-2001 (1,067), GI.2-1999 (2,139), GI.3-1999 (800), and GI.4-2000 (1,081) ( $P > 0.05$ , KW test)

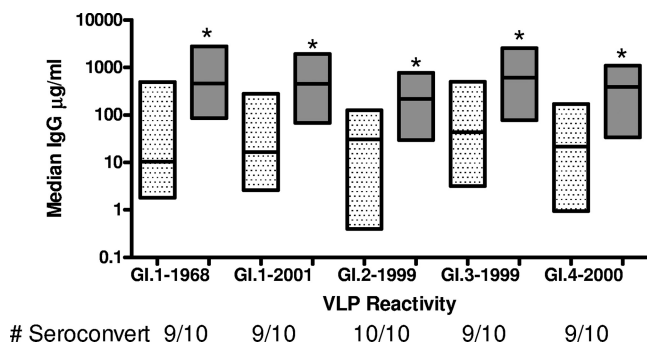


FIG. 7. GI VLPs share common antibody epitopes. The median geometric mean titer of anti-VLP IgG ( $\mu\text{g/ml}$ ) in prechallenge (day 0; dotted bars) and convalescent (day 14; shaded bars) serum samples collected from volunteers infected with GI.1-1968 and the number of subjects who seroconverted to each VLP were determined. The median titer is indicated by the line in the box. The upper and lower boundaries of the box represent the maximum and minimum values. The asterisk indicates a significant increase ( $P < 0.01$ , M-W test) in titer between day 0 and day 14 samples.

(Fig. 8C). While BT50s were not statistically different between the VLPs, for all volunteers blockade of GI.1-1968 required more serum than blockade of the other GI VLPs. This difference is independent of HBGA binding signal strength as it holds for GI.1-2001 and Chiba (both equally strong HBGA binders compared to GI.1-1968) and GI.2-1999 and GI.3-1999 (both relatively weak HBGA binders). As demonstrated in prechallenge serum samples, each volunteer had a specific VLP-HBGA blockade profile. After infection, 9 out of 10 volunteers had a sufficient BT50 for GI.1-1968. Further, all of these nine volunteers also had a BT50 for the other four GI VLPs after GI.1-1968 infection. The one volunteer who did not produce a BT50 to either of the GI.1 VLPs did raise a sufficient BT50 (BT50 of  $\leq 400$ ) to GI.2-1999 and GI.3-1999 (Fig. 9, volunteer 01). Blockade of GI.2-1999, GI.3-1999, and GI.4-2000 varied by volunteer, as shown in Fig. 9. These data indicate that the GI VLPs share potential cross-neutralization epitopes. This cross-neutralization potential may be unique to the GI strains as neither NV GI.1-1968 serum nor GII.2-1976 serum blocked interaction of GII.4-1997 VLP with H type 3 (54) (BT50 of  $\leq 200$  for both serum sets) (Fig. 10).

#### NV infection induces a GI broadly reactive T-cell response.

Although previous studies of immunity in NV-infected volunteers have focused exclusively on antibody responses (37, 53), cellular immune responses are also likely to be important components of protective immunity. In support of this hypothesis, PBMCs from volunteers challenged with GII.2-1976 demonstrated significant IFN- $\gamma$  secretion from CD4 $^{+}$  T cells after GII.2-1976 VLP *in vitro* stimulation (52). To initially characterize the cellular immune response after NV infection, PBMCs were isolated from whole blood collected from NV-infected volunteers ( $n = 10$ ) and cryo-preserved for *in vitro* VLP stimulation and secreted IFN- $\gamma$  analysis by EIA. All PBMC samples responded to PMA-ionomycin stimulation with levels of IFN- $\gamma$  secretion above the upper limit of detection (data not shown), indicating that the cell populations remained responsive after harvesting. As a group, the 10 volunteers did not have a significant response to stimulation with any of the GI VLPs

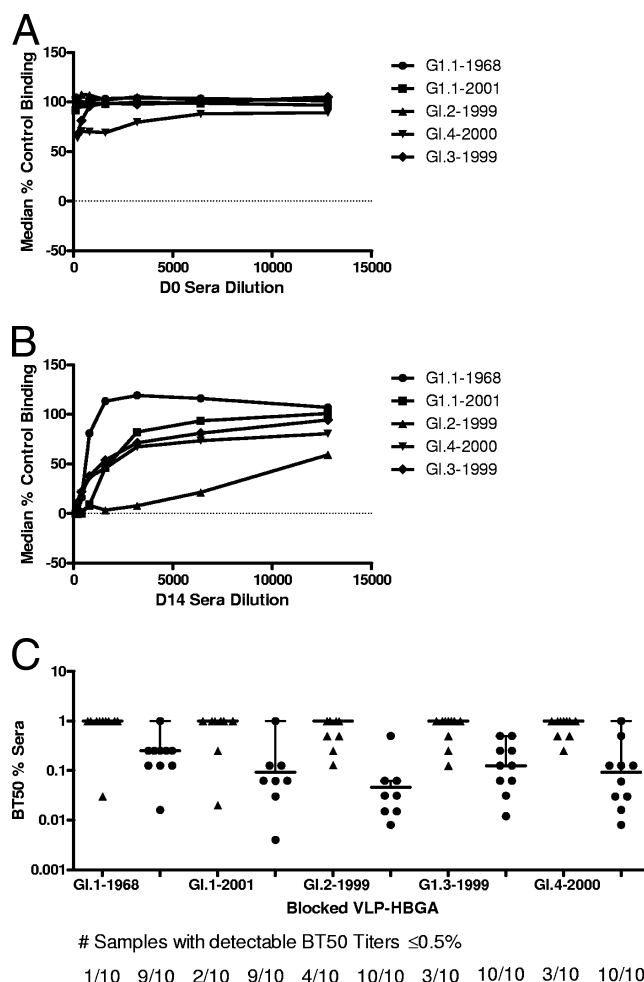


FIG. 8. Cross-blockade of GI VLPs binding to HBGA by GI.1-1968 convalescent-phase serum. Serum samples collected from GI.1-1968-infected volunteers were assayed for blockade of H type 3 interaction with GI.1-1968, GI.1-2001, and GI.2-1999 and Le $^a$  interaction with GI.3-1999 and GI.4-2000, and the median percent control binding was calculated relative to the no-serum control binding. (A) Prechallenge serum. (B) Day 14 convalescent serum. (C) Scatter plot of the median percent serum needed for BT50 and the number of volunteers with a BT50 for each VLP (indicated below the graph). The median titer is indicated by the line in the box. Error bars represent the range.

at days 4, 14, or 35 postchallenge compared to prechallenge responses (data not shown). However, individually, PBMCs from 6 of the 10 volunteers did respond with IFN- $\gamma$  secretion to at least one GI VLP stimulation (Fig. 11). Three volunteers did not have prechallenge samples for comparison. Therefore, PBMCs were considered responsive to a VLP if two consecutive postchallenge samples had a  $\geq 4$ -fold increase above the earliest collected sample (day 0 or day 4) (94). Interestingly, only two volunteers' PBMCs responded to GI.1 VLP (volunteers 09 and 10). These two volunteers also had higher prechallenge anti-GI.1 IgG and blockade titers. Of note, volunteer 10 had insufficient PBMCs for heterologous VLP stimulation; thus, potential cross-VLP responses were not determined. Three volunteers each responded to only one GI VLP, with one responder each to GI.2-1999, GI.3-1999, and GI.4-2000. The remaining volunteer responded to both GI.2-1999 and

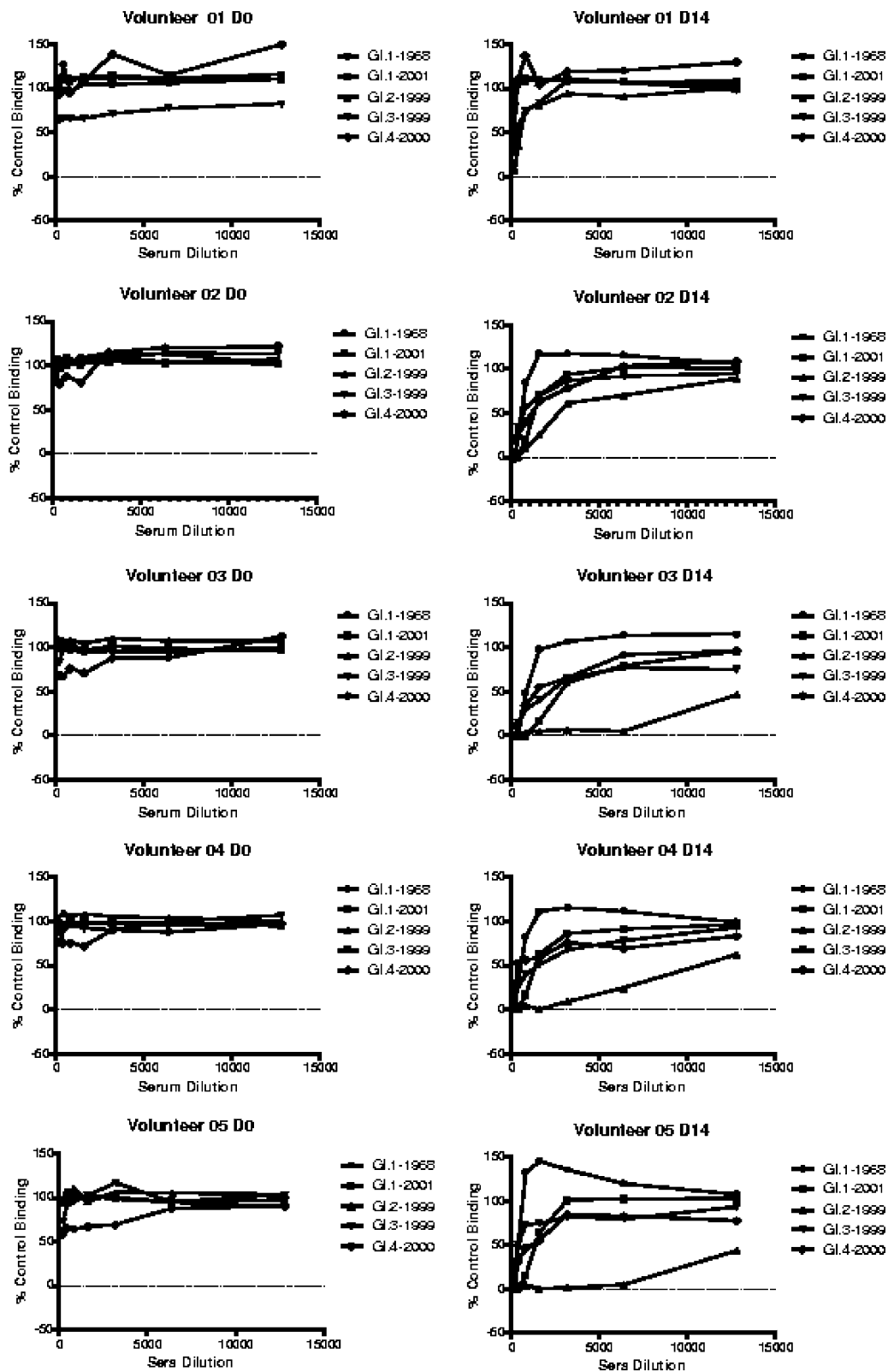


FIG. 9. GI VLP blockade responses vary by individual. Serum samples collected from GI.1-1968-infected volunteers at day 0 (prechallenge; left-hand column) and day 14 (right-hand column) were assayed for blockade of H type 3 interaction with GI.1-1968, GI.1-2001, and GI.2-1999 and Le<sup>a</sup> interaction with GI.3-1999 and GI.4-2000, and the percent control binding was calculated relative to the no-serum control binding.

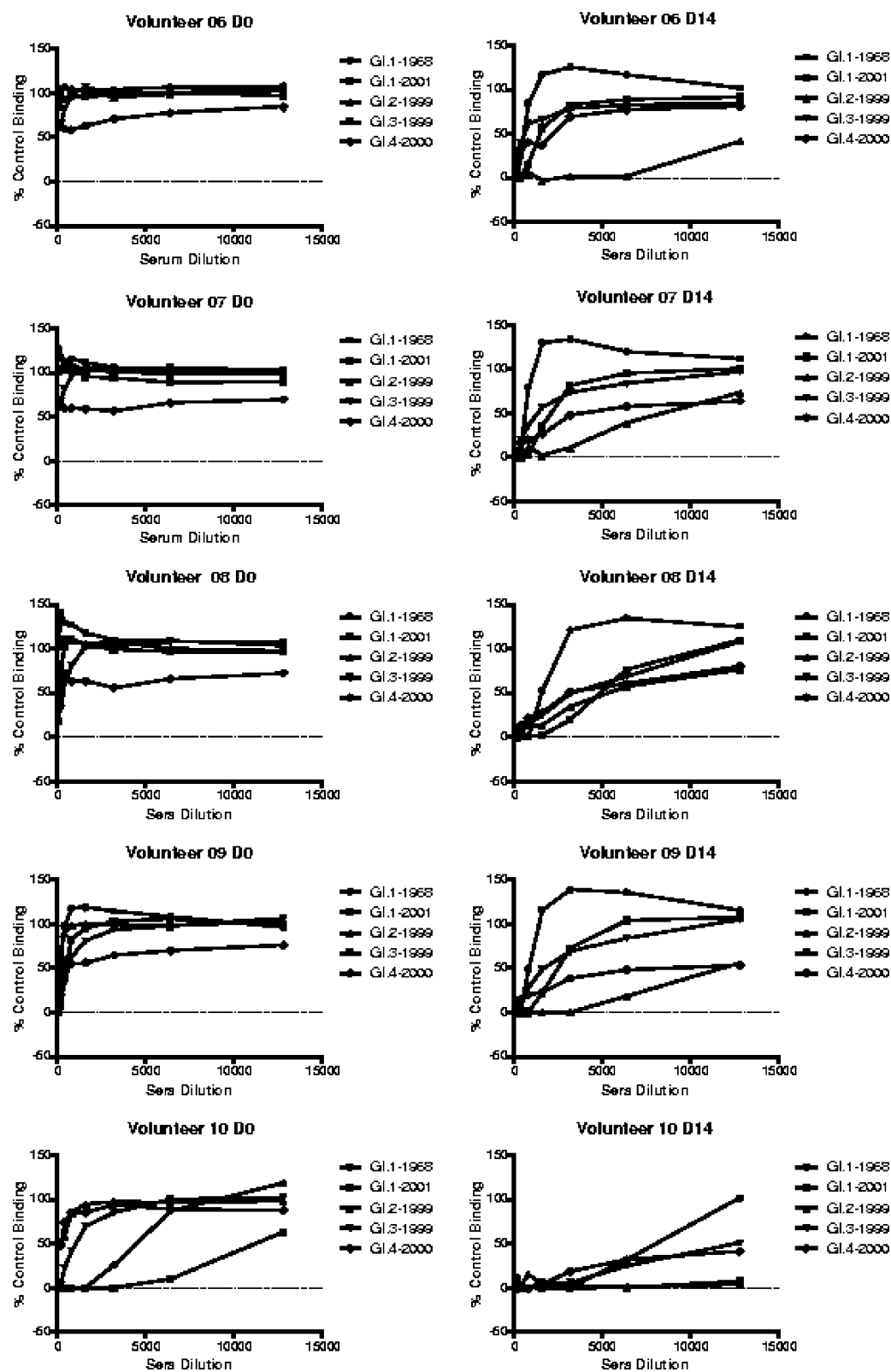


FIG. 9. —Continued.

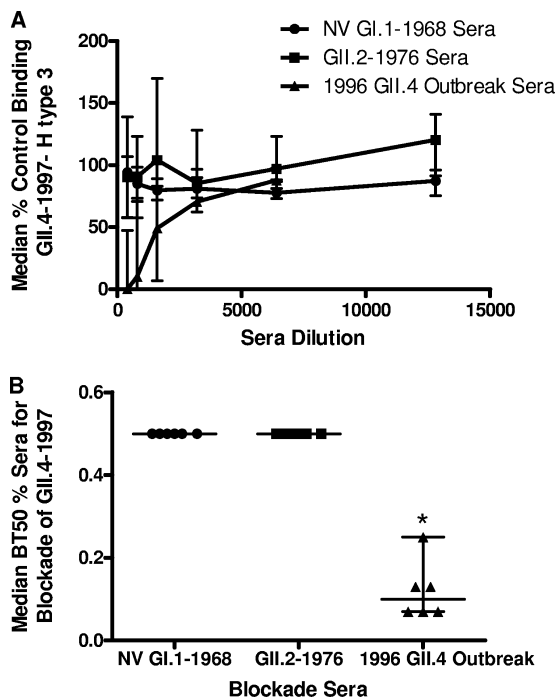


FIG. 10. GI.1-1968 convalescent-phase serum does not block cross-genogroup GII.4-1997 VLP binding to HBGA. (A) Convalescent-phase serum samples collected from individuals infected with GI.1-1968 (circles), GII.2-1976 (squares), and GII.4 (triangles) noroviruses ( $n = 6$  each strain) were assayed for blockade of GII.4-1997-H type 3 interaction, and the median percent control binding was calculated relative to the no-serum control binding. Error bars represent the range. (B) Scatter plot of the median percent serum needed for BT50 for blockade of GII.4-1997-H type 3 interaction. The median titer is indicated by the line in the box. Error bars represent the range. Serum BT50 responses significantly different ( $P < 0.05$ , KW test) from GI.1-1968 blockade are marked with an asterisk.

GI.3-2000. These data indicate that in 66% of the GI.1-1968-infected volunteers, the cellular immune response is preferentially targeting an alternative GI VLP rather than the infecting strain.

## DISCUSSION

Noroviruses are a leading cause of acute infectious gastroenteritis (45) and cause an estimated 23,000,000 infections annually in the United States (61). Although the majority of reported norovirus outbreaks are associated with the GII.4 strains, GI strains are more frequently detected in environmental monitoring studies, indicating that these strains are also circulating in the general population although disease patterns in the young and in settings of endemicity are less well understood (7, 21, 33). The fundamental mechanisms underlying frequency, severity, and/or susceptibility to repeat infection with GI and GII strains remains largely unknown, complicating vaccine design and our understanding of evolutionary and epidemiologic factors affecting disease occurrence and distribution in human populations. The continued prominence of the GII.4 strains has been attributed to evolution within the P2 subdomain of these strains that leads to receptor switching and antigenic drift, resulting in both access to previously resistant

populations and evasion of protective immunity in previously susceptible populations (23, 54). Molecular mechanisms governing continued persistence of other noroviruses in human populations are not well understood.

Bioinformatic analysis of the GI genogroup indicated that evolution within the different GI genotypes induced only minor effects on the capsid structure, which is in agreement with work conducted by Choi et al. that showed the HBGA binding sites were conserved between GI genotypes (19). The only GI genotype that contained divergent evolution was the GI.3 Desert Shield genotype, which clustered into two subclades with very little divergence within them (Fig. 1). Although this may reflect more limited sampling of the genogroup 1 biota and less sequence information available for analysis, these findings are in stark contrast to the GII genogroup, where many genotypes can be further subdivided into clusters that evolve from one another. For example, a recent bioinformatics study of GII.2 strains suggested that this genotype may be evolving new strains in a time-ordered manner over a 12-year period. It appears that the novel strains were defined by targeted variation at select residues within the capsid sequence (40). This variation primarily occurred in the surface-exposed P2 subdomain, suggesting that heterogeneity in the GII.2 strains was mediated by an immune response-driven evolution (40). In addition, similar studies with the GII.3 and GII.4 genotypes show evidence of immune response-driven evolution (12, 54).

In contrast, the primary outcome of evolution in the GI genogroup appears to be the formation of different genotypes that appear to be antigenically and structurally static. Supporting this conclusion, bioinformatic analyses of the GI.1 strains spanning 40 years did not reveal any significant evolutionary trends over time (Fig. 1 and 2). The capsid sequences of NV GI.1-1968 and GI.1-2001 are 97% similar to each other and encode only five changes within the P2 subdomain between these strains. Only one of these changes was surface exposed (D350E, Norwalk numbering) (Fig. 2) and noticeably alters the structure (D350E, Norwalk numbering) (Fig. 4). None of these changes appears to be involved in HBGA binding as GI.1-1968 and GI.1-2001 have identical carbohydrate binding profiles, with both strains dependent upon a Gal-Fuc or GalNAc for binding. This limited binding phenotype is not conserved across the GI strains as GI.2, GI.3, and GI.4 each bound to Le<sup>a</sup>, a FUT2-independent HBGA, suggesting that the FUT2 gene may not be a susceptibility allele for all GI strains (39, 53). Binding of the  $\alpha$ -(1,3/4)-fucose of Le<sup>a</sup> and to secretor-negative, Lewis-positive saliva by three of the four GI genotypes argues that the recently reported (19) predicted universal rules governing GI-HBGA epitope binding do not fully capture the phenotypic variation in HBGA binding patterns noted among different GI VLPs. These predictions, based on the structure of NV GI.1-1968 interaction with H type 1 and A trimer, argue that structural constraints within the GI strains will restrict HBGA binding patterns to either a terminal Gal-Fuc or GalNAc. Importantly, the binding site identified in the GI.1 crystallography study was almost entirely conserved in the structural models of the other GI genotype structures, with the 327-DXH-(X)<sub>48</sub>-WXSXXS-380 portion (Norwalk numbering) being strictly conserved (Fig. 2) in the GI.1 through GI.4 genotypes. This suggests that all four of the GI VLPs that we analyzed may utilize this binding site, but

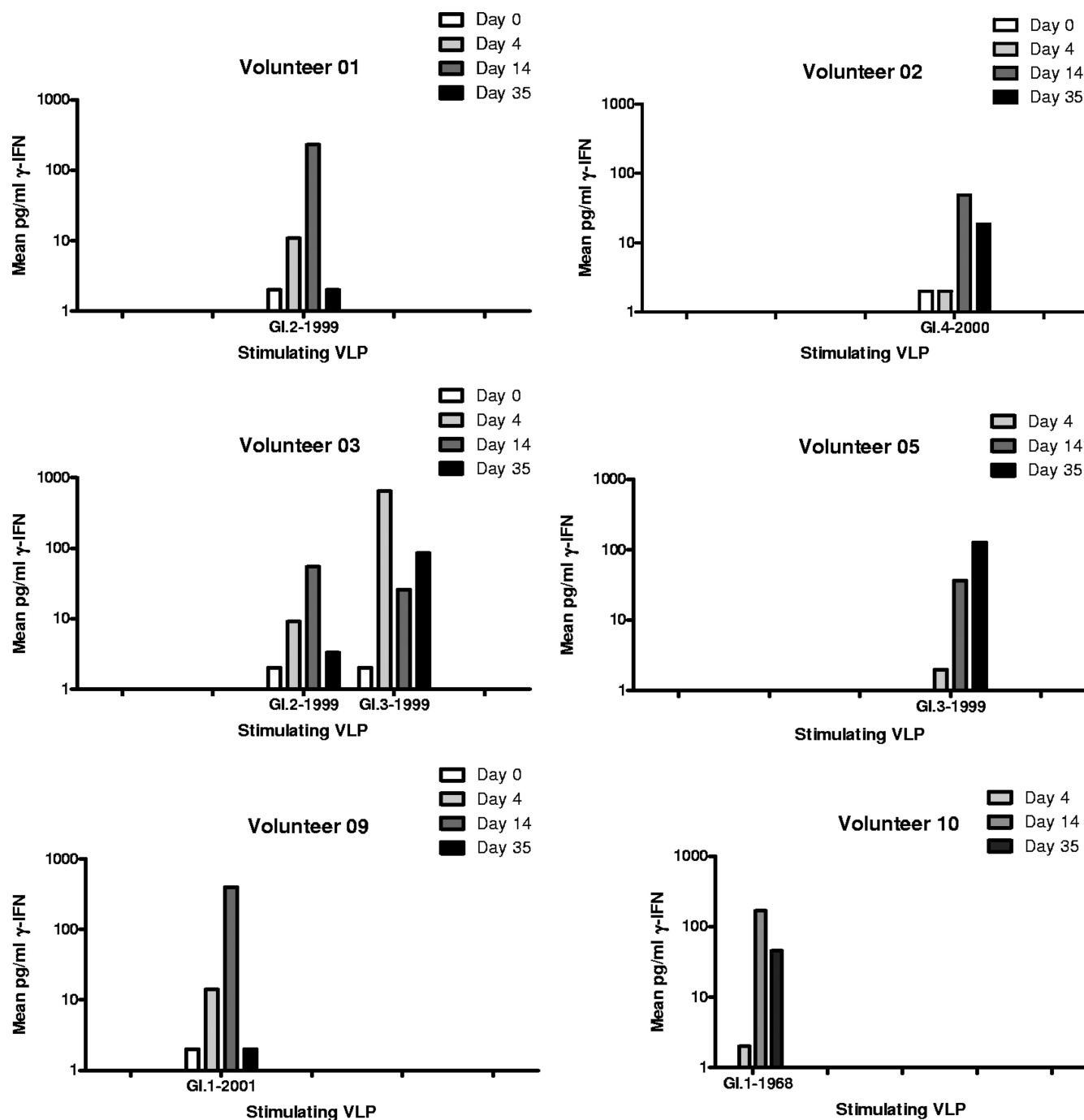


FIG. 11. GI.1-1968 infection induces GI-reactive PBMCs collected from volunteers infected with GI.1-1968 were stimulated individually with a panel of GI VLPs and IFN- $\gamma$  secretion measured by EIA on days 0, 4, 14, and 35 postchallenge. Only VLPs with two consecutive time points with elevated IFN- $\gamma$  secretion levels are shown for each volunteer.

variations in HBGA recognition may be due to the changing residue patterns in contact interface residues at positions 344, 353, 355, or, to a lesser extent, 395 (alignment numbering) or by other varying residues that compose the extended pocket around the HBGA binding sites. In addition, variation between these genotypes suggests that surface-exposed charge changes may be an important factor for determining which HBGAs can bind without steric hindrance. Moreover, a recent report has

suggested that the HBGA binding site may be conformational in nature, allowing target CHOs to fit into the structure rather than utilizing the same linear sequences for interactions (88), and therefore varying the residues distal to the binding site may be important for allowing the structural flexibility necessary for ligands to fit into the conformational pocket. Our data certainly support this possibility and argue for the need for solving additional GI VLP-CHO costructures.

While this study focused primarily on the P2 subdomain of the capsid protein, additional variation in the P1 subdomain could also contribute to differences in CHO binding, as has been suggested for GII.4 noroviruses (13, 54). Not only are crystal structures of additional GI VLPs bound to HBGA ligands needed to address these questions, but also detailed capsid mutagenesis studies may provide a means of determining the mechanisms governing variant HBGA recognition patterns in the GI noroviruses.

GI noroviruses display very complex patterns of heterotypic immune responses following infection, and many individuals are highly susceptible to reinfection. Previous studies have shown that anti-norovirus IgG is cross-reactive within a genogroup but much less between genogroups (41, 52, 64). Our results corroborate these findings (76). Using a standard VLP antigen EIA, IgG elicited by NV infection showed a high degree of reactivity to the panel of GI VLPs and a  $\geq 4$ -fold increase in titer between prechallenge and convalescent samples for each VLP. Mechanistically, this may result from the following: (i) GI.1 infection may induce high-affinity antibodies with strong heterotypic strain targeting; (ii) GI.1 infection may induce preexisting memory B cells that secrete antibody with low affinity to NV, but higher affinity to other GI strains; or (ii) a conserved, surface-exposed region of the capsid protein necessary for capsid function may provide a common epitope to which cross-reacting antibodies bind.

This confirmation of cross-reactivity among GI clusters has several implications. First, coupled with the data demonstrating that GI strains bind to FUT2-independent molecules, the cross-reactivity provides an explanation for previously reported observations that secretor-negative volunteers have serum antibodies that react to NV GI.1-1968 (49, 52, 53). Second, the high degree of cross-strain reactivity emphasizes the limitation of using VLP antigens in antibody detection antigen EIAs as diagnostic tools in outbreak investigations. While a  $\geq 4$ -fold increase in anti-VLP titers between acute and convalescent samples is predictive of norovirus infection (52, 53), there was not a significant difference between mean prechallenge or convalescent IgG titers in a comparison of the response to NV GI.1-1968 to any of the other VLPs. These data support previous findings of a high degree of antibody cross-reactivity between GI strains (64) and emphasize that the determination of an outbreak strain by EIA reactivity to a limited panel of VLPs is imprecise. Third, the data suggest that even an expanded panel of VLPs may not be able to do more than differentiate between a norovirus genogroup I versus II infection. This is an important issue in the context of outbreak investigation, where we have found subjects to be more willing to donate serum than stool samples for analysis and longitudinal epidemiological studies that use sera collected over time to draw conclusions about the epidemiology of endemic norovirus infections (84). Lastly, these data also suggest that conserved antigenic epitopes among GI strains likely exist, arguing for the need for the development of comprehensive monoclonal antibody panels for epitope mapping.

In contrast to the VLP EIA, we have shown that the antibody blockade of the VLP-HBGA assay (30) is able to discriminate antigenic differences between different strains of GII.4 noroviruses (54). Surprisingly, this high degree of strain specificity was not observed for the GI strains, where human

convalescent antisera cross-blocked not only different strains within the same genotype but also strains between different GI genotypes. This observation supports structural analysis indicating that the GI strains lack the significant heterogeneity in the P2 domain observed in GII.4 strains (19, 54). Even though the prechallenge sera had reactive IgG to each of the GI VLPs by EIA, the prechallenge sera from 8 out of 10 volunteers was not able to block the interaction of any of the VLPs with HBGA, possibly because of insufficient titer or lack of binding site specificity. However, with one exception, after NV GI.1-1968 infection, serum IgG was capable of blocking VLP-HBGA binding for each GI VLP. Similar concentrations of serum were needed to block all five GI VLPs binding to HBGA, indicating that infection with GI.1-1968 may result in production of potentially cross-genotype neutralizing antibody. Additional antibodies with protective potential are likely to be present in polyclonal sera. It is likely that protection from infection is not solely dependent upon antibodies capable of inhibiting the VLP-HBGA interaction. Further study of antibody epitopes and of the role of soluble capsid protein in antibody interactions is needed. Although as a group the sera blocked all of the GI VLPs tested, each volunteer exhibited a distinct pattern of strain blockade. This variable pattern of neutralization is similar to that identified in humans vaccinated with strains of influenza virus where neutralizing antibody responses varied by individual, with all developing a neutralizing antibody response but some developing greater increases in neutralization titer to strains other than the vaccination strain (8, 28, 73).

Overall, little information is available regarding the role of T cells in norovirus infection. Initial characterization of the GII norovirus-induced T-cell response identified a predominantly Th1 CD4-dependent cellular response characterized by significant IFN- $\gamma$  secretion (52). This study supports these findings with a GI norovirus strain as IFN- $\gamma$  was secreted in significant amounts after NV GI.1-1968 infection in most volunteers. Six out of 10 of the infected volunteers with PBMC sample sets for analysis responded to *in vitro* stimulation to at least one GI VLP. Surprisingly, only two had a  $\geq 4$ -fold increase in IFN- $\gamma$  production between early and late PBMCs in response to GI.1 stimulation (85- and 200-fold increase above baseline comparator) in contrast to results shown for PBMCs collected after GII.2-1976 infection, where five out of seven infected volunteers responded to homologous VLP stimulation (median fold increase above baseline, 33.2). Further, detected overall IFN- $\gamma$  secretion levels were lower in this study (median fold increase of peak, 148.4 pg/ml compared to 1,418 pg/ml in GII.2-1976-infected volunteers). Possible explanations for this observation included timing of the postchallenge sample collection (days 0, 8, and 21 for the GII.2-1976 study compared to days 0, 4, 14, and 35 here), and timing of postsample processing (immediately after blood collection compared to  $\sim 24$  h after collection with shipment at uncontrolled temperatures in the intermediate). In particular, the delay required for shipment has been demonstrated to reduce PBMC activity (11). Importantly, four out of five volunteers with sufficient PBMCs for cross-reactivity studies responded more robustly to other GI VLPs. This broad PBMC response pattern is indicative of the response to cross-reactive epitopes presented under different human leukocyte antigen (HLA) backgrounds.

Both the PBMC reactivity and antibody blockade data indicate that complex patterns of adaptive GI cross-reactive immune responses may provide some level of cross-protection. Moreover, data demonstrating that both antibody and T-cell responses of some volunteers are skewed away from the infecting strain and directed toward other GI strains reveal a potential mechanism of deceptive imprinting (original antigenic sin [OAS]), suggesting that complex preexposure histories may complicate GI immune responses and allow for repeat infections, which highlights a potential complication in the design of efficacious norovirus vaccines. The skewed B-cell responses in some of the GI.1-1968-infected volunteers may be explained by two mechanisms. In the first mechanism, memory cells may generate antibodies to the current challenge strain that are also cross-reactive with other strains. A pan-anti-GI antibody response would explain the high degree of IgG cross-reactivity and blockade between the strains but not the difference in blockade between the strains for individuals. The second mechanism, the concept of OAS, can explain both of these observations and has been described both in mice (5, 48) and in humans (62, 93) at the antibody and T-cell levels although this is controversial (95) as it represents a highly variable response regulated by complex antigenic relationships, host genetics, and population exposure histories. OAS is a host-mediated response that induces memory cells to produce antibody to a previous, similar, infecting pathogen instead of generating a primary response to a slightly antigenically different, current, infecting pathogen. OAS results in the production of lower-affinity antibody and T cells for the current infection than what would be produced as the result of a primary immune response (63). An OAS effect depends upon an individual's epitope presentation and, thus, HLA type, the current infecting strain, and exposure history to other strains (5, 63). While speculative, these factors, and the complex antigenic interactions between strains, may result in either cross-protection or attenuation of protective immune responses as a function of prechallenge exposure histories. Evidence of both skewed B- and T-cell responses in some GI.1-1968-infected volunteers highlights the need for continued human challenge studies both to unravel the complex norovirus immune response and to evaluate norovirus infection as a potential model system for study of OAS.

Early human NV GI.1-1968 rechallenge studies (69, 96) have suggested three potential outcomes to NV rechallenge: resistance to infection on the first and subsequent challenge, development of protective immunity after infection, and repeated susceptibility to infection. Our contemporary NV GI.1-1968 challenge study demonstrated that about 20% of challenge volunteers are secretor-negative individuals who lack FUT2 and are genetically resistant to NV GI.1-1968 infection (53). We also identified about 40% of the susceptible secretor-positive individuals who remained uninfected even after high doses of NV GI.1-1968 challenge (53). An early mucosal IgA response was associated with these uninfected susceptible volunteers, indicating that a protective anti-norovirus immune response to NV GI.1-1968 infection might occur following exposure in some individuals. Although additional studies are needed, the work presented in this report suggests that the third group of volunteers, those who are susceptible to repeat NV GI.1-1968 infection, may be unable to mount robust protective immune responses because of preexposure biases and

OAS. The existence of this group of individuals has been cited in the literature as proof that long-term protective immunity to norovirus infection is not possible, a hypothesis in conflict with data gathered in contemporary NV GI.1-1968 (53) and GII.2-1976 (52) challenge and outbreak investigations (80, 81). Although speculative, these studies provide one possible explanation of why some volunteers can become repeatedly infected with NV GI.1-1968 (69, 96). Although correlation with human disease is unknown, new studies with mouse norovirus (MNV) indicating that infection with high but not low levels of MNV results in a lack of protective immunity (55) suggest the possibility of altered immune regulation following some norovirus infections.

We have articulated a potential model to explain the variable immune responses and susceptibility patterns noted following GI.1 infection, recognizing that this model is largely based on descriptive findings following challenge with an ~40-year-old norovirus isolate. It is not clear whether challenges with contemporary GI.1 isolates will reflect these infection outcome patterns or whether similar findings would occur following challenge with other isolates. However, given the remarkable stability of GI strains demonstrated here, these findings are likely applicable to contemporary strains. The model clearly defines a need for additional GI and GII rechallenge studies that exclude genetically resistant volunteers and use modern diagnostic assays, such as the antibody VLP-HBGA blockade, large panels of GI and GII VLPs, the isolation of human monoclonal antibodies following infection, and a variety of T-cell assays, to empirically determine the underlying mechanisms governing variant GI.1-1968 infection outcomes and to evaluate the hypothesis that OAS may induce the immune response toward other norovirus strains. Further studies should include higher numbers of infected and uninfected volunteers and consider comparing antibody and T-cell responses to antigens outside the major capsid protein as well as alternative measures of virus neutralization besides receptor blockade if a cell culture system for noroviruses becomes available. Given the strict requirements for human challenge studies, the gnotobiotic pig model for human norovirus GII.4 infection may also serve as an important model and/or provide critical information about strain cross-reactivity and cross-protection (15, 83). However, human rechallenge studies remain key to understanding the fundamental mechanisms governing susceptibility to and frequency of norovirus infection.

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